Capsid Sequence Diversity in Small Round Structured Viruses From Recent UK Outbreaks of Gastroenteritis

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Genetic typing of small round structured viruses (SRSVs) by reverse transcription–polymerase chain reaction (RT-PCR) and sequencing has been confined to analysis of the RNA polymerase because of the considerable genome variability outside of this region. To provide capsid sequence data for epidemiological studies and outbreak investigations, a broadly reactive capsid PCR was developed using two sets of degenerate, inosine-containing primers. Primer pairs CapIa/CapIb and CapIIa/CapIIb specifically amplify a 223-bp region of the SRSV capsid open reading frame from SRSV genetic groups I and II, respectively. The capsid PCR was used to investigate SRSVs from nine UK outbreaks of gastroenteritis occurring between 1992 and 1995. Differential amplification by the primer pairs suggested that three strains belonged to genetic group I and six to genetic group II. The capsid amino acid sequences of the group I strains were 75.9% to 79.3% identical with Sot/91/UK (group I), while those of the group II strains were 75.9% to 98.3% identical with Bri/93/UK (group II). Phylogenetic comparison of the capsid region from the outbreak strains and 13 previously characterised SRSVs revealed clusters of strains closely related to Bri/93/UK and Tor/77/C within genetic group II. With the exception of some Bri/93/UK-like strains, there was no correlation between capsid sequence and the geographical origin of SRSVs. UK strains were found with greater than 90% capsid sequence identity to SRSVs from various locations worldwide including Australia (Cam/94/A), Canada (Tor/77/C), Hawaii (Haw/71/US), and Saudi Arabia (DSV395/90/SA) together with group I (B447/92/UK) and group II (Yat/94/UK) strains that were genetically distinct from known SRSV capsids. Three SRSVs very closely related to Bri/93/UK were from recent UK hospital outbreaks. These Bri/93/UK-like strains appear to be prevalent in the UK. ***J. Med. Virol. 52:14–19, 1997.*** © 1997 Wiley-Liss, Inc.

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INTRODUCTION

Small round structured viruses (SRSVs) cause acute diarrhoea and vomiting in children and adults. The high infectivity of the virus results in outbreaks in semi-closed communities such as hospitals with frequent secondary person-to-person spread [Caul, 1994]. SRSVs possess single-stranded positive sense RNA genomes of between 7,500 nt and 7,700 nt and are classified as members of the Caliciviridae [Cubitt et al., 1995]. The complete genomes of three strains have been characterised: the UK Southampton (Sot/91/UK) [Lambden et al., 1993] and Lordsdale (LDV/93/UK) [Dingle et al., 1995] viruses and the prototype US Norwalk virus (Nor/68/US) [Jiang et al., 1993], revealing similar genome organisations with three overlapping open reading frames (ORFs). The large 5′ ORF1 encodes a polyprotein containing helicase, protease, and polymerase domains. ORF2 codes for the single capsid protein of about 58 kDa, and there is a small 3′ ORF3 of unknown function. The complete capsid sequences of ten further strains have been determined: the UK Bristol (Bri/93/UK) [Green et al., 1994] and Melksham (Mel/89/UK) [Green et al,. 1995a] viruses, Toronto virus (Tor/77/C) [Lew et al., 1994c], Hawaii virus (Haw/71/US) [Lew et al., 1994b], Desert Shield virus (DSV395/90/SA) [Lew et al., 1994a], Mexico virus (MXV/89/M) [Jiang et al.,

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1995a], the Japanese strains KY-89/89/J and OTH-25/89/J [Wang et al., 1994], and the recently reported Australasian strains Camberwell (Cam/94/A) [Cauchi et al., 1996] and Auckland (Auc/NZ) (Regli et al., unpublished).

Comparison of SRSV sequences has revealed considerable variation which is greatest in ORF2 and ORF3 [Green et al., 1994; Lew et al., 1994b; Wang et al., 1994]. Phylogenetic analysis of these data also showed that this variation was not continuous and that SRSVs can be divided into two distinct genetic groups. Group I contains Sot/91/UK and Nor/68/UK, while group II includes LDV/93/UK, Bri/93/UK, Mel/89/UK, Tor/77/C, and Haw/71/US. Morphologically classical human caliciviruses were found to have a genome organisation distinct from that of SRSVs [Liu et al., 1995].

The genetic diversity of SRSVs has hindered the development of diagnostic polymerase chain reactions (PCRs). Initially, PCR primers based on a single SRSV strains were able to amplify only a limited range of isolates. Primers based within the RNA polymerase were most broadly reactive as this region exhibited least genome variation and contained amino acid motifs conserved among SRSVs. Subsequently, PCR detection rates have been improved by designing primers capable of amplifying SRSVs from both genetic groups [Green et al., 1994; Green et al., 1995c], including degenerate inosine-containing primers [Green et al., 1995b].

Studies of SRSV genetic variation have relied upon sequence analysis of PCR amplicons from the RNA polymerase region [Green et al., 1993; Ando et al., 1994; Jiang et al., 1995b; Norcott et al., 1994; Moe et al., 1994; Wolfaardt et al., 1995]. Differentiation of strains by Southern hybridisation using probes based on different SRSV antigenic types has also been reported [Ando et al., 1995b]. Lack of sequence conservation outside of the polymerase region has prevented further routine characterisation of strains, although there may be insufficient variation within the polymerase region to enable closely related strains to be differentiated [Ando et al., 1995a].

To provide more detailed molecular epidemiological data, we designed two sets of broadly reactive, inosine-containing primers that amplify part of the SRSV capsid ORF from both genetic groups. The primers are also group specific, allowing rapid initial genetic grouping of strains by the PCR. Application of these primers to examine capsid sequence variation among SRSVs from recent UK outbreaks of gastroenteritis is presented.

MATERIALS AND METHODS

Faecal Samples

Faecal samples were collected from outbreaks of gastroenteritis occurring within the UK between 1992 and 1995 and stored at 4°C. The following outbreaks have been previously described: B447/92/UK and B453/92/UK [Green et al., 1994], and Lym/94/UK [Green et al., 1995b]. Additional outbreaks examined were B449/92/UK (Carmarthen, Dyfed), Car/94/UK (Cardiff, South Glamorgan), Yat/94/UK (Yate, Avon), S031/95/UK (Lymington, Hampshire), S017/94/UK, S020/94/UK, S033/95/UK, and S039/95/UK (all Southampton, Hampshire). SRSVs were initially detected in faecal samples by negative staining EM.

RNA Extraction and cDNA Synthesis

Viral RNA was extracted from faecal samples using TRIzol™ reagent (GIBCO BRL) and single-stranded cDNA synthesised as described previously [Lambden et al., 1993] using random hexamers.

Primers

Primers CapIa (5′-^{5644}CICAAATGTAIAATGGYTGGGT^{5665}-3′) and CapIb (5′-^{5866}TGIIARAGIACATTICIWACATCYTC^{5841}-3′) were designed using the following genetic group I SRSV sequences (accession numbers are in parentheses): Sot/91/UK (L07418), Nor/68/US (M87661), DSV395/90/SA (U04469), and KY-89/89/J (L23828). Nucleotide co-ordinates refer to the Sot/91/UK genome. Primers CapIIa (5′-^{5362}CIAGAATGTAIAAYGGKTATGC^{5383}-3′) and CapIIb (5′-^{5584}TGIIAGAAAITRTTICIRACATCWGG^{5559}-3′) were designed using the following genetic group II SRSV sequences (accession numbers are in parentheses): Bri/93/UK (X76716), Mel/89/UK (X81879), Tor/77/C (U02030), Haw/71/US (U07611), and OTH-25/89/J (L23830). Nucleotide co-ordinates refer to the LDV/93/UK (X86557) genome. Primer binding sites were selected such that the 3′ terminal sequences were different from the corresponding genetic group I and II primers. It has been shown that the PCR is very sensitive to 3′ end primer:template mismatches [Huang et al., 1992], and it was hoped that this would enable each of the primer pairs to specifically amplify SRSVs from only one genetic group. Primers GLPSG2 and YGDD1 have been described previously [Green et al., 1995b].

PCRs and Sequencing

PCRs were performed as described previously [Green et al., 1994] using 35 cycles of 94°C for 20 sec, 50°C for 20 sec, and 72°C for 10 sec. PCR amplicons were purified from agarose gels by binding to a silica matrix (Geneclean II, BIO101) and sequenced directly using the PCR primers as described previously [Green et al., 1994]. Polymerase amplicons were also sequenced as clones in M13mp18. Phylogenetic analysis of sequence data was performed using the DNASTAR LaserGene software.

Nucleotide Sequence Accession Numbers

Sequence data have been deposited in the EMBL/Genbank data library and assigned the accession numbers Z73989 to Z73999.

RESULTS

Comparison of the N-terminal coding regions of genetic group I and II SRSV capsid ORFs identified semi-conserved sequences that enabled two pairs of degenerate, inosine-containing PCR primers to be designed. The primer binding sites were selected such that CapIa/

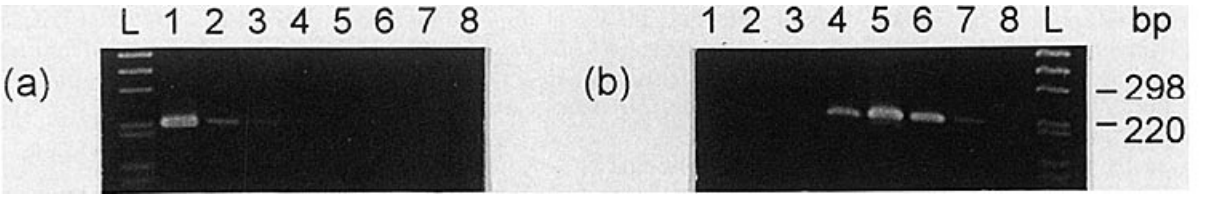

Fig. 1. Agarose gels showing 223bp amplicons generated by the PCR using primer pairs **(a)** CapIa/CapIb and **(b)** CapIIa/CapIIb with SRSV cDNA from outbreaks attributed to genetic groups I or II. Group I outbreaks: **lane 1**—Sot/91/UK, **lane 2**—B447/92/UK, **lane 3**—B453/92/UK. Group II outbreaks: **lane 4**—Bri/93/UK, **lane 5**—Lym/94/UK, **lane 6**—Mel/89/UK, **lane 7**—B449/92/UK. In **lane 8,** water was substituted for cDNA as a PCR negative control. Lanes marked **L** contain 1 μg of a DNA ladder with fragment sizes shown in bp.

CapIb would preferentially amplify group I SRSVs, while CapIIa/CapIIb would preferentially amplify group II SRSVs, allowing rapid initial grouping of strains by the PCR. Both sets of primers generate a 223-bp amplicon. The ability of the primers to discriminate between group I and II SRSVs was tested using two genetic group I strains (B447/92/UK and B453/92/UK) and two group II strains (B449/92/UK and Lym/94/UK) which had been previously characterised by sequence analysis of the polymerase region only. The group I strain Sot/91/UK and group II strains Bri/93/UK and Mel/89/UK for which the capsid sequences are already known were included as positive controls. The PCR results (Fig. 1) show that the two sets of primers are each able to specifically amplify SRSVs from different genetic groups.

EM-positive faecal samples from seven previously uncharacterised UK outbreaks occurring between 1992 and 1995 were tested with the capsid primers and amplicons obtained from five of the outbreaks. Differential amplification by the two sets of primers suggested that one of these outbreak strains (Car/94/UK) belonged to genetic group I while the other four (S031/95/UK, S033/95/UK, S039/95/UK, and Yat/94/UK) belonged to genetic group II (Table I). To confirm the genetic group designations predicted by the PCR, the nine new capsid amplicons were sequenced and compared with the representative group I and group II strains, Sot/91/UK and Bri/93/UK, respectively (Table I). The group I strains were most closely related to Sot/91/UK (75.9–79.3% amino acid identity), while the group II strains were more closely related to Bri/93/UK (75.9–98.3% amino acid identity) confirming the PCR results. For the isolates B447/92/UK, B449/92/UK, B453/92/UK, and Lym/94/UK, the results corresponded with the group designations based on their polymerase sequences. Three strains from recent outbreaks occurring in Hampshire (S031/95/UK, S033/95/UK, and S039/95/UK) were very closely related to Bri/93/UK (Table I). S033/95/UK and S039/95/UK , isolated from outbreaks at Southampton General Hospital, possessed identical capsid sequences (95.4% nucleotide identity with Bri/93/UK). SO31/95/UK, isolated from an outbreak at Lymington Hospital, differed from the SO33/SO39 strain at one nucleotide position (94.9% nucleotide identity with Bri/93/UK).

Using primers of GLPSG2 and YGDD1, polymerase amplicons were obtained for the two outbreaks (S017/92/UK and S020/92/UK) that had been negative for the PCR using the capsid primers, demonstrating that the faecal samples did not contain RT-PCR inhibitors. Sequence analysis revealed that these outbreak strains were very similar group II SRSVs and were distantly related to Mel/89/UK (75.0% amino acid identity).

TABLE I. Comparison of SRSV Genetic Groups for Strains From Nine Recent UK Outbreaks of Gastroenteritis as Predicted by Differential Capsid PCR*

SRSV strain	Genetic group predicted by the PCR	% amino acid identity for capsid amplicons	
		Sot/91/UK	Bri/93/UK
B447/92/UK	I	79.3	60.3
B453/92/UK	I	75.9	58.6
Car/94/UK	I	77.6	58.6
B449/92/UK	II	53.4	75.9
Lym/94/UK	II	55.2	75.9
S031/95/UK	II	55.2	98.3
S033/95/UK	II	55.2	98.3
S039/95/UK	II	55.2	98.3
Yat/94/UK	II	53.4	84.5

*The derived amino acid identities between capsid PCR amplicons and the equivalent capsid regions of representative group I and group II strains Sot/91/UK and Bri/93/UK, respectively, are shown.

To investigate the genetic relatedness of the outbreak strains to SRSVs of known capsid type, the derived amino acid sequences from the capsid PCRs were compared with the corresponding regions from those strains for which the complete ORF2 sequences are available (Fig. 2a). Phylogenetic analysis of this region of the SRSV capsid (Fig. 2b) clearly shows the two genetic groups with amino acid identity between group I and group II ranging from 51.7% to 63.8% while viruses in group I are 74.1% to 96.6% identical and those within group II are 69.0% to 100% identical. The group I strains B453/92/UK and Car/94/UK are most closely related to DSV395/90/SA with 93.1% and 91.4% identity, respectively, while B447/92/UK is more distantly related to Sot/91/UK and Nor/68/US with about 80% identity. The three group II Bri/93/UK-like strains possess the same amino acid sequence and differ from Bri/93/UK by only a conservative valine-to-isoleucine substitution. The other group II strains Lym/94/UK and B449/92/UK are closely related to Haw/71/US (98.3% identity) and Tor/77/C (98.3% identity), respectively, while Yat/94/UK is more distantly related to Bri/93/UK (84.5% identity).

SRSV Capsid Sequence Diversity

(a)

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Sot/91/UK       GNMRVRILLAGNAFSAGKIIVCCVPPGFTSSSLTIAQATLFPHVIADVRTLEPIEMPL
Nor/68/US       .......M......T......S.I....G.HN.....................D...V..
KY-89/89/J      .......M......T......S.I....G.QQ.....................D...V..
B447/92/UK      ......VV......T...V.I.......Q.RT.S.................D.V.V..   Group I
Car/94/UK       .....KV.......T.....IS.I......Q.IS...M.M.........V.....I..
DSV395/90/SA    .....KV.......T.....IS.I....AAQNIS.....M.........V.....V..
B453/92/UK      ...K..V.......T.....IS......AAQNVS.....M.........V.....V..

Bri/93/UK       GGFEVQVILAGNAFTAGKVIFAAVPPNFPTEGLSPSQVTMFPHIIVDVRQLEPVLIPL
LDV/93/UK       ............................................................
Cam/94/A        ............................................................
S031/94/UK      ................I...........................................
Yat/94/UK       ..V..E.V.........I..........P.N.....I..L.............R...
Tor/77/C        .......V.........I....I.....IDN..AA.I..C..V.........INL.M
B449/92/UK      .......V.............I.....IDN..AA.I..C..V.........INL.M   Group II
Auk/NZ          .......V.............I.....IDN..AA.I..C..V.........INL.M
OTH-25/89/J     .......V........A.....I.....IDN..AA.I..C..V.........INL.M
MXV/89/M        .......V........A.I....I.....IDN..AA.I..C..V.........INL.M
Mel/89/UK       ..M....M.........LV......H..V.N...QKI.....V.I...T.....L..
Haw/71/US       ..V....L.........LV...I..H..L.N...G.I.....V.I...T.....L..
Lym/94/UK       ..V....L.........LV...I..H..I.N...G.I.....V.I...T.....L..
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(b)

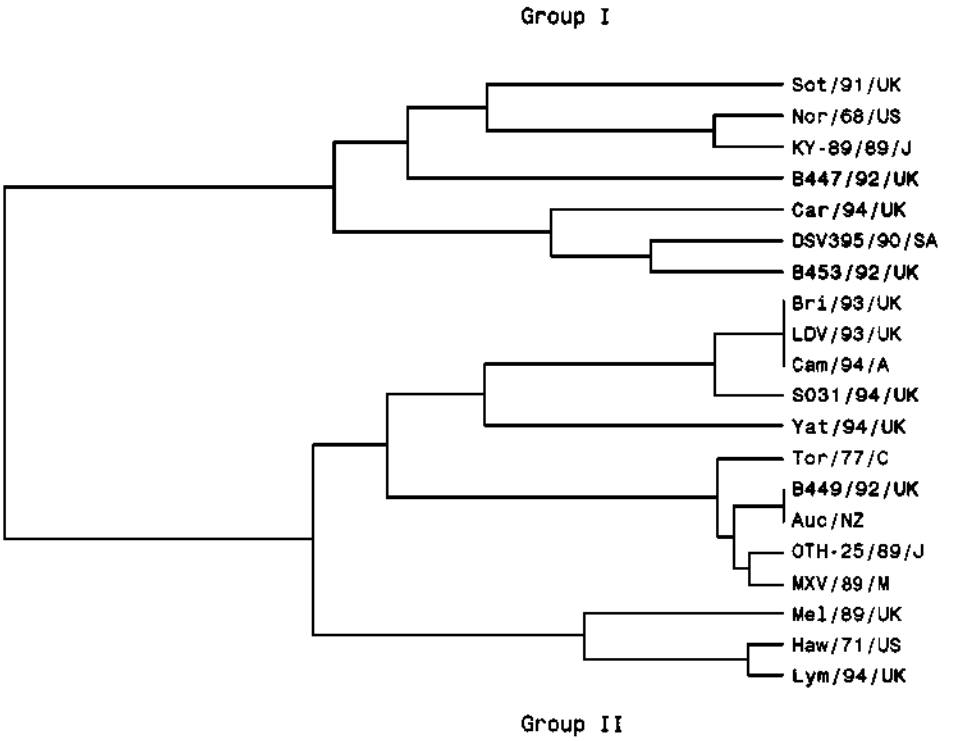

Fig. 2. **a:** Multiple alignments of group I and group II capsid PCR amino acid sequences from the nine UK outbreak strains (highlighted in bold type) together with the equivalent regions from 13 further strains for which the complete capsid sequences are available. The two genetic groups are indicated and dots represent residues in group I and group II sequences that are identical with Sot/91/UK and Bri/93/UK, respectively. S031/94/UK represents S033/94/UK and S039/94/UK, which have identical amino acid sequences. The accession numbers for previously reported stains are given in Materials and Methods with the exception of Auc/NZ (U46039), Cam/94/A (U46500), and MXV/89/Mex (U22498). **b:** Phylogenetic representation of the sequences shown in (a). The two SRSV genetic groups are indicated.

DISCUSSION

Molecular investigations of SRSV-associated outbreaks of gastroenteritis have provided detailed epidemiological information that would have been impossible using classical diagnostic techniques. The extreme variability of the virus has enabled strains to be identified by sequence "fingerprints" obtained from PCR amplicons, typically from the relatively well-conserved RNA polymerase region. This technique has been used to confirm that related outbreaks were caused by the same strain of SRSV [Green et al., 1995b] and to trace the source of an outbreak back to an infected individual [Kilgore et al. 1996]. To provide additional sequence data from the structural region of the SRSV genome, we have developed the first broadly reactive capsid PCR using sets of primers that are specific to each of the two SRSV genetic groups. This allows initial rapid grouping of new strains by the PCR with sequence analysis of the capsid amplicon providing more detailed information about capsid types within a genetic group.

The capsid PCR was used to analyse SRSVs from nine recent UK outbreaks of gastroenteritis. Differential amplification of the viral cDNA by the two pairs of primers identified three group I and six group II strains. The genetic groups designated by the PCR were subsequently confirmed by sequence analysis. UK strains were identified with capsid sequences closely related to SRSVs from geographically diverse countries such as Australia (Cam/94/A), Canada (Tor/77/C), Hawaii (Haw/71/US), and Saudi Arabia (SDV395/90/SA), together with group I (B447/92/UK) and group II (Yat/94/UK) strains with sequences distinct from previously characterised capsids. Tor/77/C-like strains appear to have a global distribution, with isolates having greater than 96% amino acid identity to Tor/77/C within the capsid region being found in Japan, Mexico, New Zealand, and the UK. These may represent similar endemic strains or imported strains that may have a common origin.

Three Bri/93/UK-like strains were isolated from hospital outbreaks occurring in Hampshire, UK between September 1994 and June 1995. A strain with 99.8% capsid amino acid identity to Bri/93/UK (LDV/93/UK) had previously been isolated in Hampshire in 1993 [Dingle et al.,1995]. S031/95/UK, S033/95/UK, and S039/95/UK have also been shown to be antigenically Bri/93/UK-like using an antigen enzyme immunoassay based on antisera to recombinant LDV/93/UK capsid (E. Pelosi, personal communication). The high mutation rate among single-stranded RNA viruses such as SRSVs can result in a virus strain existing as a population of related quasispecies [Steinhauer and Holland, 1987]. The three Bri/93/UK-like strains may represent endemic Bri/93/UK quasispecies that have emerged as the virus continued to circulate in the community. Other groups have isolated Bri/93/UK-like viruses within the UK [Ando et al., 1995b], suggesting that this is a prevalent SRSV type, while genetic group I SRSVs appear to be less common [Green et al., 1995c].

The capsid primers were able to amplify a wide range of SRSV capsid sequences from 9/11 (82%) of the outbreaks examined. The two strains not amplified by the capsid PCR (S017/92/UK) and S031/92/UK) possessed group II polymerase sequences that were very similar to each other but distinct from those of the established group II strains used to design CapIIa and CapIIb. This suggested that S017/92/UK and S031/92/UK would have new capsid sequences not recognised by the group II primers. If distinct SRSV capsid types are defined as having greater than 20% nucleotide sequence divergence, complete sequences are available for only three group I (Sot, DSV, and Nor/KY) and four group II (Bri/Cam/LDV, Mel, Haw, and Auc/MXV/OTH/Tor) capsid types. Future refinement of the capsid primers to broaden their reactivity will be possible when new SRSV capsid types are identified and characterised.

The capsid PCR amplifies a region of ORF2 encoding the N-terminal part of the capsid. As the main site of variation between antigenically distinct strains is a central hypervariable region [Green et al., 1995a] and as in Nor/68/US, monoclonal antibody binding sites have been mapped to the C-terminal half of the capsid [Hardy et al., 1996], it is unlikely that the capsid PCR product encodes significant SRSV antigenic determinants. Hence, genetic relatedness between strains within this region may not correspond to antigenic relatedness. However, the phylogenetic relationships between the capsid PCR sequences and the complete capsid sequences of nine strains [Green et al., 1995a] are similar, suggesting that comparison of strains within this region (which represents about 11% of the total capsid ORF) is a good predictor of overall capsid identity. Comparison of available antigenic data for SRSVs is complicated by the lack of a common typing system. Further studies will be necessary to determine the relationship between SRSV genetic and antigenic variation and the global epidemiology of SRSV genetic types.

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